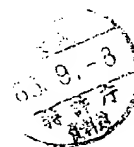INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

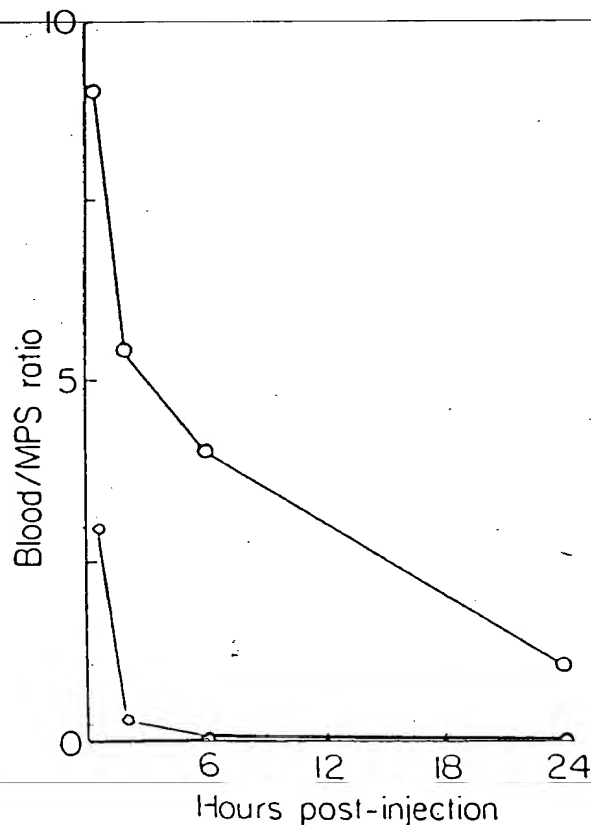| (51) International Patent Classification⁴ : A61K 9/42, 9/52, B01J 13/02 C07F 9/10	A1	(11) International Publication Number: WO 88/04924
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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
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(54) Title: LIPOSOMES WITH ENHANCED CIRCULATION TIME

(57) Abstract

A composition of liposomes which contain an entrapped pharmaceutical agent and are characterized by (a) liposome sizes predominantly between about 0.07 and 0.5 microns; (b) at least about 50 mole percent of a membrane-rigidifying lipid, such as sphingomyelin or neutral phospholipids with predominantly saturated acyl chains; and (c) between about 5-20 mole percent of a glycolipid selected from the group consisting of ganglioside GM_1, saturated phosphatidylinositol, and galactocerebroside sulfate ester. The liposomes in which a drug is entrapped are administered intravenously in a suspension as therapeutic drug treatment, and the liposomes have enhanced circulation time in the bloodstream.

PCT

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-1-

5   LIPOSOMES WITH ENHANCED CIRCULATION TIME

1.   Field of the Invention
          The present invention relates to liposome
therapeutic compositions, and particularly to liposomal
10   formulations which have enhanced circulation time in the
bloodstream, when administered intravenously.

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3.   Background of the Invention

        Liposome delivery systems have been proposed
for a variety of drugs. For use in drug delivery via
the bloodstream, liposomes have the potential of provid-
ing a controlled "depot" release of a liposomeentrapped
drug over an extended time period, and of reducing toxic
side effects of the drug, by limiting the concentration
of free drug in the bloodstream. Liposome/drug composi-
tions can also increase the convenience of therapy by
allowing higher drug dosage and less frequent drug
administration. Liposome drug delivery systems are
reviewed generally in Poznansky et al.

        One limitation of intravenous liposome drug
delivery which has been recognized for many years is the
rapid uptake of blood-circulating liposomes by the mono-
nuclear phagocytic system (MPS), also referred to as the
reticuloendothelial system (RES). This system, which
consists of the circulating macrophages and the fixed
macrophages of the liver (Kupffer cells), spleen, lungs,
and bone marrow, removes foreign particulate matter,
including liposomes, from blood circulation with a half
life on the order of minutes (Saba). Liposomes, one of
the most extensively investigated particulate drug car-
riers, are removed from circulation primarily by Kupffer
cells of the liver and to a lesser extent by other mac-
rophage populations.

        A variety of studies on factors which effect
liposome uptake by the RES have been reported. Early
experiments, using heterogeneous preparations of

multilamellar liposomes (MLV) containing
phosphatidylcholine (PC) and cholesterol (CH) as their
principal lipid constituents, demonstrated that these
liposomes are rapidly removed from circulation by uptake
into liver and spleen in a biphasic process with an ini-
tial rapid uptake followed by a slow phase of uptake
(Gregoriadis, 1974; Jonah; Gregoriadis, 1972; Juliano).
Half-time for removal of MLV from circulation was on the
order of 5-15 min. following intravenous (IV) injection.
Negatively charged liposomes are removed more rapidly
from circulation than neutral or positively charged
liposomes. Small unilamellar liposomes (SUV) are
cleared with half-lives approximately three-to four-fold
slower than MLV (Juliano; Allen, 1983). Uptake of
liposomes by liver and spleen occurs at similar rates in
several species, including mouse, rat, monkey, and human
(Gregoriadis, 1974; Jonah; Kimelberg, 1976; Juliano;
Richardson; Lopez-Berestein).

        Liposomes which are capable of evading the RES
would have two important benefits. One is the increased
liposome circulation time in the blood, which would both
increase the pharmacokinetic benefits of slow drug
release in the bloodstream, and also provide greater
opportunity for tissue targeting where the liver,
spleen, and lungs are not involved. The second benefit
is decreased liposome loading of the RES. In addition
to the role of the RES in removing foreign particles,
the RES is involved in several other functions, includ-
ing host defense against pathogenic microorganisms, par-
asites, and tumor cells, host responses to endotoxins
and hemorragic shock, drug response, and responses to
circulating immune complexes (Saba, Altura). It is
important, therefore, in liposome administration via the

bloodstream, to avoid compromising the RES seriously, by massive short-term or accumulated liposome uptake.

One approach which has been proposed is to increase liposome circulation time by increasing liposome stability in serum. This approach is based on studies by one of the inventors and others which have shown that factors which decrease leakage of liposome contents in plasma also decrease the rate of uptake of liposomes by the RES (Allen, 1983; Gregoriadis, 1980; Allen, 1981; Senior, 1982). The most important factor contributing to this effect appears to be bilayer rigidity, which renders the liposomes more resistant to the destabilizing effects of serum components, in particular high density lipoproteins (Allen, 1981; Scherphof). Thus, inclusion of cholesterol in the liposomal bilayer can reduce the rate of uptake by the RES (Gregoriadis, 1980; Hwang; Patel, 1983; Senior, 1985), and solid liposomes such as those composed of distearoylphosphatidylcholine (DSPC) or containing large amounts of sphingomyelin (SM) show decreased rate and extent of uptake into liver (Allen, 1983; Ellens; Senior, 1982; Hwang).

However, this approach appears to have a limited potential for increasing liposome circulation times in the bloodstream. Studies carried out in support of the present invention, and reported below, indicated that 0.1-0.2 micron liposomes containing optimal membrane-rigidifying liposome formulation are predominantly localized in the RES two hours after intravenous liposome administration. Although longer circulation times are achieved with small unilamellar vesicles or SUVs (having a size range between about 0.03 and 0.08 microns), SUVs are generally less useful in drug delivery due to their smaller drug-carrying capacity, and

their greater instability, which can lead to rapid release of liposome-associated drug, and to liposome fusion events that produce large and heterogeneous-size liposomes.

Several investigators, including the applicants, have also explored the possibility of increasing liposome circulation times by designing the liposome surface to mimic that of red blood cells. The role of cell surface carbohydrates in cellular recognition phenomena is widely appreciated (Ashwell, Hakomori, Karlsson). The chemistry, metabolism, and biological functions of sialic acid have been reviewed (Schauer). Surface sialic acid, which is carried by gangliosides, and glycoproteins such as glycophorin, plays an important role in the survival of erythrocytes, thrombocytes, and lymphocytes in circulation. Enzymatic removal of sialic acid, which exposes terminal galactose residues, results in rapid removal of erythrocytes from circulation, and uptake into Kupffer cells of the liver (Durocher). Desialylation of thrombocytes (Greenberg) and lymphocytes (Woodruff) also results in their rapid removal by the liver.

Although desialylated erythrocytes will bind to Kupffer cells or peritoneal macrophages _in vitro_ in the absence of serum, serum must be added in order for significant phagocytosis to occur. The nature of the serum components mediating endocytosis is speculative, but immunoglobin and complement (C3b) are thought to be involved. Czop et al. (Czop) have shown that sheep erythrocytes, which are not normally phagocytosed by human monocytes, will bind C3b and be phagocytosed upon desialylation. Okada et al. (Okada) have demonstrated that sialyglycolipids on liposome membranes restrict activation of the alternative complement pathway and

that removal of the terminal sialic acid from the
glycolipids abolishes this restricting capacity and
results in activation of the alternative complement
pathway. Sialic acid, therefore, may be functioning as
a non-recognition molecule on cell membranes partly
through its ability to prevent binding of C3b, thus pre-
venting phagocytosis via the alternative complement
pathway. Other immune factors may also be involved in
liposome phagocytosis. Alving has reported that 50% of
the test sera from individual humans contain naturally
occurring "anti-liposome" antibodies which mediated
complement-dependent immune damage to liposomes.

The observations reported above suggest that
surface sialic acid, and/or other red-cell surface
agents, incorporated into liposomes, for example, in the
form of ganglioside or glycophorin, may lead to
increased circulation half-lives of liposomes. This
approach is described, for example, in U.S. Patent No.
4, 501, 728 for "Masking of Liposomes from RES Recogni-
tion", although this patent does not disclose whether
significant RES masking is actually achieved by coating
liposomes with sialic acid.

In fact, experiments conducted in support of
the present applications indicate that sialic acid, in
the form of gangliosides, has a limited ability to
extend circulation half lives _in vivo_ in liposomes which
are predominantly composed of conventional liposomes
lipids, such as egg phosphatidylcholine (egg PC) or egg
PC:cholesterol mixtures. _In vivo_ uptake studies on
PC:cholesterol:ganglioside liposomes (0.2 microns or
smaller) indicate that the injected liposomes are local-
ized predominantly in the RES two hours post
administration.

In summary, several approaches for achieving enhanced liposome circulation times in the bloodstream have been proposed. Heretofore, however, the approaches have produced quite limited improvements in blood circu-
5   lation times, particularly in liposomes in the 0.07-0.2 micron size range which are generally most desirable for parenteral drug compositions.

4.   Summary of the Invention
10   It is therefore one general object of the present invention to provide an improved liposome composition which gives significantly improved blood circulation times.

15   A more specific object of the invention is to provide such a composition in which liposomes are predominantly localized in the bloodstream, rather than in the liver and spleen, several hours after liposome administration.

20   Yet another object of the invention is to provide such a composition containing liposomes which are in a selected size range between about 0.08-0.2 microns.

Providing a method for significantly extending the lifetime of drug-carrying liposomes in the bloodstream, for improved drug delivery and/or drug tar-
25   geting in tumor treatment is still another object of the invention.

The invention includes a liposome composition which is designed for enhanced circulation in the bloodstream. The liposomes in the composition are char-
30   acterized by: (a) liposome sizes in a selected size range between about 0.07 and 0.4 microns; (b) substantially homogenous-phase liposome bilayers composed of at least about 50 mole percent of a membrane-rigidifying lipid, and (c) between about 5-20 mole percent of a

glycolipid component selected from the group consisting of ganglioside GM₁, hydrogenated phosphatidylinositol, and sulfatide, i.e., sulfate esters of lactocerebrocidesmonogalactosyl. The lipophilic moiety of the glycolipid serves to anchor the component in the liposome bilayers, without phase separation therein.

The liposomes show enhanced tissue distribution, as measured by the ratio of liposomal marker present in the blood to the combined amount of marker in the liver and spleen, when measured 2, 4, and 24 hours after intravenous liposome administration. Preferably, blood/RES ratio, when measured 2 hours after administration, is substantially greater than the sum of the blood/RES ratios obtained with similarly constructed liposome compositions containing in one case, at least about 50 mole percent of the membrane rigidifying lipid, but not the glycolipid, and in the other case, between 5-20 mole percent of the glycolipid, but not the membranerigidifying lipid.

In one preferred embodiment, the membrane-rigidifying lipid is a combination of sphingomyelin and a neutral phospholipid, such as phosphatidylcholine (PC), in a preferred molar ratio of between about 2:1 and 4:1. In another embodiment, the lipid is a saturated PC, with or without cholesterol.

The method of the invention is designed for extending the bloodstream lifetime of drug-containing liposomes which are administered intravenously in a suspension of liposomes whose sizes are in a selected size range between about 0.07-0.4 microns. The method includes preparing the liposomes to contain (a) at least about 50 mole percent of a membrane-rigidifying lipid in the liposome bilayers, which are substantially phase-homogeneous, and (b) between about 5-20 mole

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percent of a glycolipid component selected from the group consisting of ganglioside GM_1, hydrogenated phosphatidylinositol, and sulfatide.

The method gives significantly enhanced drug uptake in tumors, when administered intravenously to a tumor-bearing subject. The method is therefore useful in administering an anti-tumor compound for the treatment of the tumor by intravenous administration.

These and other objects and features of the invention will become more fully apparenet when the following detailed description of the invention is read in conjunction with the accompany figures.

Brief Description of the Figures

Figure 1 shows the time course of decrease of blood/RES ratios in a test subject injected IV with (1) liposomes containing ganglioside GM_1, but not SM (solid circles) and (2) with liposomes containing both GM_1 and SM (open circles);

Figures 2A and 2B show the effect of increasing molar amounts of GM_1 on percent injected liposome marker in blood (circles), liver (triangles), and spleen (squares) two hours post injection, in liposomes containing either PC:CH, 2:1 (A) or SM:PC, 4:1 (B); and

Figures 3A and 3B show linear regression plots of different liposome formulations for (A) liposomal marker in the RES versus the marker in the bloodstream, and (B) liposomal marker in the J6456 tumor (solid triangles) and B-16 tumor (solid circles) versus amount in the bloodstream; and

Figure 4 shows the glycolipids ganglioside GM_1 (A), hydrogenated phosphatidylinositol (B) and monogalactosyl stearate sulfatide (C) which are components of the liposome compositions of the invention.

Detailed Description of the Invention

The liposome composition of the invention is
designed for delivering a drug or other agent, such as
nutritional supplements, vitamins, or chelated metal, to
a subject via the bloodstream, and for relatively slow
uptake of the liposomes by the RES, allowing the drug or
agent to be released from the liposomes into the
bloodstream over an extended period of several hours or
more. Alternatively, the composition is designed, by
appropriate surface modification of the liposomes, for
targeting via the bloodstream to non-RES target tissues,
to allow the drug or agent to concentrate in the immedi-
ate region of the target tissue.

Section IA below describes the general method
used to evaluate liposome uptake by the RES in vivo,
section IB, the combination of liposome components which
have been found, according to one aspect of the inven-
tion, to give high blood circulation times for intrave-
nously injected liposomes, and section IC, methods for
preparing, sizing, and sterilizing drugcontaining
liposomes designed for intravenous administration. The
utility of the liposome composition, in drug delivery
and drug targeting, is discussed in Section II.

I. Preparing the Liposomal Composition

A. Measuring liposome uptake by the RES in vivo

The method used for evaluating liposome circu-
lation time in vivo measures the distribution of intra-
venously injected liposomes in the bloodstream and the
primary organs of the RES at selected times after injec-
tion. In the standardized model which is used, RES
uptake is measured by the ratio of total liposomes in

the bloodstream to total liposomes in the liver and
spleen, the principal organs of the RES. In practice,
age and sex matched mice are injected intravenously (IV)
through the tail vein with a radiolabeled liposome com-
5   position, and each time point is determined by measuring
total blood and combined liver and spleen radiolabel
counts, and in many studies, complete dissection, weigh-
ing, and radioactivity determinations of all body parts
was done. Total blood counts are calculated by assuming
10   that the total blood volume makes up 7% of the animal's
body weight. Experimental methods are detailed in Exam-
ple 2.

       Since the liver and spleen account for nearly
100 % of the initial uptake of liposomes by the RES, the
15   blood/RES ratio just described provides a good approxi-
mation of the extent of uptake from the blood to the RES
in vivo. For example, a ratio of about 1 or greater
indicates a predominance of injected liposomes remaining
in the bloodstream, and a ratio below about 1, a predom-
20   inance of liposomes in the RES. For most of the lipid
compositions of interest, blood/RES ratios were calcu-
lated at 2, 4, and 24 hours.

       The data obtained with the model animal system
can be reasonably extrapolated to humans and veterinary
25   animals of interest. This is because, as mentioned
above, uptake of liposomes by liver and spleen has been
found to occur at similar rates in several mammalian
species, including mouse, rat monkey, and human
(Gregoriadis, 1974; Jonah; Kimelberg, 1976; Juliano;
30   Richardson; Lopez-Berestein). This result likely
reflects the fact that the biochemical factors which
appear to be most important in liposome uptake by the
RES--including opsinization by serum lipo proteins,
size-dependent uptake effects, and cell shielding by

surface moieties are common features of all mammalian
species which have been examined.

B.   Lipid Components

5   The lipid components used in forming the
liposomes of the present invention are selected to meet
three important criteria:

           First, a major portion of the lipids, i.e.,
more than 50 mole percent, are neutral
10 membrane-rigidifying components, by which is meant
uncharged lipid components which produce relatively
rigid, close-packed lipid bilayer structures. Typi-
cally, the lipids are predominantly saturated lipids
whose phase transition temperature (T_C) is above about
15 25°C, and preferably between about 30°C and 50°C. Pre-
ferred membrane-rigidifying lipids include SM (T_C about
30°C) and neutral phospholipids, such as PC whose acyl
chains are predominantly saturated. One saturated PC
which has been investigated extensively herein is
20 distearoyl PC (DSPC) whose T_C is about 50°C.

           Secondly, and according to an important fea-
ture of the invention, the lipid components must contain
a negatively charged glycolipid which has a single nega-
tive charge which appears to be partially shielded,
25 i.e., inaccessible to certain types of charge-charge
binding interactions, on the outer surface of the
liposomes. To date, three glycolipids which have this
feature, as evidenced by high blood/RES ratios achieved
with the liposome foirmulation, have been identified.
30 These are ganglioside GM_1, hydrogenated
phophatidylinositol (HPI), and sulfatides, i.e., sulfate
esters of galactocerebrosides. These three compounds
are shown in Figures 1A-1C, respectively, where the R
group in the GM_1 and sulfate compounds are long-chain

hydrocarbons.   The particular sulfatide shown in the
figure is cerebronic acid.

Thirdly, the above lipid components must pro-
duce a substantially homogeneous-phase bilayer struc-
ture, by which is meant that the critical membrane
rigidifying and negatively charged lipid components
exist in a single phase, as opposed to discrete phases
which are physically separate.   As will be seen, this
requirement both enhances blood/RES values ochievable
with the liposomes, and also minimizes rapid liposome
breakdown and leakage in the bloodstream.

The importance of membrane-rigidying lipid
components in combination with one of the selected
glycolipids can be appreciated from blood/RES data pre-
sented in Examples 3, 4, 8 and 9.   Table 1 in Example 3
shows several liposome compositions, and corresponding
blood/RES values measured 2 hours post injection.   A
comparison of compositions 3 and 4 in the table shows
the large increase in blood/RES values which are
obtained by addition of cholesterol to liposomes con-
taining egg PC and GM_1.   Cholesterol is known to have a
rigidifying or "packing" effect on relatively fluid lip-
ids, such as egg PC, which has a T_c of about 0°C.   More
importantly, for purposes of the present invention, a
comparison of compositions 3 and 4 with compositions 12
and 13 indicate that GM_1 in combination with SM gives
much higher blood/RES values than the ganglioside in
combination with PC.   Here it is noted, with reference
to compositions 17 and 18, that cholesterol decreases
blood/RES ratios in $SM:GM_1$ formulations, presumably
because of the known fluidizing effect of cholesterol in
some rigid-lipid compositions.

The effect of subsituting DSPC, i.e., a
saturated-chain PC, for egg PC in some of the Table 1

formulations is seen in Table 2 from Example 4. Clearly
DSPC and GM_1 together effectively increase blood/RES
values, an effect which is enhanced still further by
addition of SM to the compositions, with highest ratios

5   being observed with the highest relative amounts of
SM:PC. Note that cholesterol appears to have less of a
fluidizing effect, as evidenced by blood/RES ratios, in
the presence of DSPC.

           The effect of saturated lipids on blood/RES

10   values achievable with HPI is seen in Table 5 and 6,
where compositions containing PI (unsaturated PI) in
PC:CH and HPI in unsaturated PC (DOPC):CH are compared
with compositions containing HPI:DSPC:CH. The blood/RES
ratio of the saturated composition is substantially

15   higher than that of the unsaturated compositions (either
unsaturated PI or PC). Note also that the saturated
composition is more stable in the bloodstream than the
two compositions containing unsaturated PI or PC, as
evidenced by the greater total recovery after 24 hours.

20   The requirement for saturated PI, e.g., HPI, is to pre-
vent phase separation of the PI component in the
rigid-lipid bilayer structure.

           The effect of saturated lipids on the
blood/RES ratio of sulfatide containing liposomes can be

25   appreciated from a comparison of the blood/RES data in
Table 6. As seen, the blood/RES ratio at 24 hours
increases from about 15 fold when SULF is used in combi-
nation with saturated lipid components. As defined
herein, sulfatide includes ios

30   Example 8 examines blood/RES ratios in several
liposome compositions 4 hours post injection. The
results are consistent with the above results seen at 2
hours post injection: liposomes containing GM_1, when
combined with DSPC and CH, in the presence or absence of

SM, give much higher blood/RES values than in liposomes containing egg PC plus CH. The data here also indicate that HPI, in the presence of saturated phospholipid (DSPC) and cholesterol, also gives high blood/RES

5   values.

Table 6 from Example 9 shows blood/RES values for a number of liposome compositions, as measured 24 hours post injection.- The compositions are arranged according to increasing blood/RES values. It is appar-

10 ent first that blood/RES values have declined signifcantly between 4 and 24 hours, but that the best formulations still show relatively good blood/RES values, e.g., 0.3 or higher, after 24 hours. These formulations include SULF, HPI, and GM_1 in combination with

15 saturated PC (DSPC) plus cholesterol, in the rpresence or absence of SM.

Considering now the glycolipid components of the liposomes, the above-discussed data, and particularly the comparative data from Table 6, indicate that

20 GM_1, HPI, and SULF are all effective, in combination with membrane-rigidifying components, in producing high blood/RES ratios. Studies conducted in support of the invention compare these three glycolipids, and their effect on blood/RES ratios, with a variety of related

25 glycolipids or phospholipids.

Table 3 from Example 7 shows blood/RES values for PC:CH and SM:PC compositions containing one of a variety of different glycolipids and negatively charged phospholipids. The abbreviations and pertinent struc-

30 tural features of the glycolipids are detailed in Example·7. Of the several lipid components which were investigated only GM_1 or GM_1 in combination with GAL or GLU gave high blood/RES values in PC:CH liposomes. In

SM:PC liposomes, SULF gave a relatively high blood/RES value.

The blood/RES values of SM:PC liposomes containing one of several different gangliosides, as detailed in Example 7, was also investigated. As seen from Table 4, removing one or more end sugar groups from GM_1 (GM_2 and GM_3), removing the sialic acid residue form GM_1 ($ASGM_1$), or the presence of more than one sialic acid residue (GD_{1a} or GT_{1b}) substantially eliminates the blood/RES enhancement effect of GM_1.

In Table 5, it is seen that GM_1, SULF, and HPI all give comparable blood/RES values (about 1) in PC:CH liposome, whereas GM_1 and HPI (SULF was not tested) gave highest values with rigid-lipid components. These three components gave the highest blood/RES values in saturated lipid formulations, as noted above.

Figures 2A and 2B are plots of blood/RES values as a function of GM_1 mole ratio in two different liposome formulations. The first, shown in Figure 2A, is a PC:CH formulation (which gives suboptimal blood/RES ratios), and the second, an SM:PC formulation. As discussed in Example 6, only the latter formulation shows a strong GM_1 effect. As seen, the optimal concentration of GM_1 is between 5-15 mole percent. The effect of high glycolipid concentration on blood/RES ratios is seen in Example 10, which examines 4 and 24 hour blood/RES ratios for liposomes containing DSPC:CH or PC;CH and increasing molar concentrations of HPI. Molar ratios of HPI above about 25% substantially eliminated the enhanced blood/RES values seen at concentrations of about 16 mole percent or below.

The results discussed above suggest that the glycolipids which are most effective in enhancing blood/RES values, in combination with

membrane-rigidifying components, share two features.
The first is a single negative charge, which may be
associated with sialic acid, as in GM_1, with a phosphate
polar group, as in HPI, or with a terminal galactosyl
group, as in sulfate. One possible role of the charged
group is to reduce liposomal aggregation, either _in
vitro_ or _in vivo_. None of the uncharged glycolipids,
including CTRI, DGDG, MGDG, GAL, GLOB, $ASGM_1$, or GLU,
and none of the glycolipids which were doubly or triply
charged, gave high blood/RES ratios.

        A second requisite feature appears to be
charge shielding which may prevent direct protein and/or
calcium-ion interactions with the glycolipid charged
group. In the case of GM_1, the charged sialic group is
shielded potentially by the two terminal sugar groups
(see Figure 4A). Interestingly, GM_2, which has one
potential shielding sugar residue, does not enhance
blood/RES values, suggesting that both sugar residues
are required. In HPI, the negatively charged phosphate
group is shielded by a terminal inositol residue. This
is in contrast to PA where the charged phosphate acid
group would be expected to be exposed, and PG, where the
charged phosphate group is shielded by a relatively
small glycerol residue. Here it is noted that PG, which
does have some charge shielding, gives blood/RES values
which are intermediate between HPI and PA. In the case
of sulfatide glycolipids, the relatively high blood/RES
values indicate that the negatively charged sulfate
group on the galactosyl residue (Figure 4C) is partially
buried or shielded on the liposome surface. By con-
trast, cholesterol sulfate, which would be expected to
have exposed sulfate groups, gives low blood/RES values.
The apparent requirement for charge shielding may also
explain why doubly and triply charged glycolipids, such

as GD_{1a} and GT_{1b} give relatively poor blood/RES ratios, since at least one charge would be expected to be unshielded.

The requirement that the lipid components form a homogeneous-phase bilayer is aimed, in part, at ensuring good liposome stability in the bloodstream over a period of 24 hours or longer. Bilayer phase homogeneity should also contribute to improved blood/RES values, since liposome instability would be expected to enhance RES uptake. This may explain, for example, why the $SM:GM_1$ formulation has a relatively low blood/RES ratio after 24 hours (Table 6), yet gives a very high ratio after 2 hours (Table 1). The data from both tables indicate that this formulation is quite unstable and/or leaky in the bloodstream, as detailed in Example 9.

Phase inhomogeneity may also explain why PI is less effective than HPI in combination with DSPC:CH mixtures in enhancing blood/RES ratios, since HPI would be expected to be more phase-compatible (more similar T_c) with saturated PC.

In addition to the membrane-rigidifying agents and gangliosides required in the liposome composition, the liposomes may be formulated to include other neutral vesicle-forming lipids which do not significantly compromise the RES-evasion properties of the liposomes. An obvious example is cholesterol which is used in many of the above formulations above, at a mole ratio of about 30%.

The liposomes may also include protective agents such alpha-tocopherol, or other free-radical inhibitors, to minimize oxidative damage to the liposomes and/or entrapped drug carried in the liposomes.

C.   Preparing the Liposome Composition

The liposomes may be prepared by a variety of
techniques, such as those detailed in Szoka et al, 1980.
One preferred method for preparing drug-containing
5   liposomes is the reverse phase evaporation method
described by Szoka et al and in U.S. Patent No.
4,235,871. In this method, a solution of liposome-
forming lipids is mixed with a smaller volume of an
aqueous medium, and the mixture is dispersed to form a
10   water-in-oil emulsion. The drug or other pharmaceutical
agent to be delivered is added either to the lipid solu-
tion, in the case of a lipophilic drug, or to the aque-
ous medium, in the case of a water-soluble drug. Here it
is noted that all lipid and aqueous components should
15   preferably be sterile and pyrogen free. After removing
the lipid solvent by evaporation, the resulting gel is
converted to liposomes, with an encapsulation effi-
ciency, for a water-soluble drug, of up to 50%. The
reverse phase evaporation vesicles (REVs) have typical
20   average sizes between about 2-4 microns and are predomi-
nantly oligolamellar, that is, contain one or a few
lipid bilayer shells. The method is detailed in
Example 1A.

The REVs are readily sized, as discussed
25   below, by extrusion to give oligolamellar vesicles hav-
ing a maximum selected size preferably between about
0.08 to 0.4 microns. Experiments conducted in support
of the present invention indicate that sized
oligolamellar vesicles of this type show substantially
30   higher blood/RES ratios than similar sized multilamellar
vesicles (MLVs), and that smaller REVs, e.g., 0.16-0.17
micron sizes, give higher ratios than larger REVs, e.g.,
0.4 microns. Another advantage of REVs is the high
ratio of encapsulated drug to lipid which is possible,

allowing greater drug doses to be administered in a given lipid dose.

MLVs, where desired, can be formed by simple lipid-film hydration techniques. In this procedure, a mixture of liposome-forming lipids of the type detailed above dissolved in a suitable solvent is evaporated in a vessel to form a thin film, which is then covered by an aqueous medium. The lipid film hydrates to form MLVs, typically with sizes between about 0.1 to 10 microns. These vesicles, when unsized, show relatively poor blood/RES ratios, as seen in Table 9, for the unextruded MLV composition. Typically, MLVs are sized down to a desired size range of 0.5 or less, and preferably between about 0.07 and 0.12 microns by extrusion, as detailed below.

One effective sizing method for REVs and MLVs involves extruding an aqueous suspension of the liposomes through a polycarbonate membrane having a selected uniform pore size, typically 0.05, 0.08, 0.1, 0.2, or 0.4 microns (Szoka). The pore size of the membrane corresponds roughly to the largest sizes of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane. This method of liposome sizing is used in preparing homogeneous-size REV and MLV compositions described in the examples below. A more recent method involves extrusion through an asymmetric ceramic filter. The method is detailed in U.S. patent application for Liposome Extrusion Method, Serial # 829,710, filed February 13, 1986.

Alternatively, the REV or MLV preparations can be treated to produce small unilamellar vesicles (SUVs) which are characterized by sizes in the 0.04-0.08 micron range. However, as indicated above, SUVs have a

relatively small internal volume, for delivery of water-
soluble drugs, and they tend to fuse to form larger het-
erogeneous size liposomes with heterodisperse drug leak-
age and RES uptake characteristics, and are leakier than
5   REVs or MLVs. SUVs can be produced readily by homoge-
nizing or sonicating REVs or MLVs, as described in Exam-
ple 1C.

After final sizing, the liposomes can be
treated, if necessary, to remove free (non-entrapped)
10   drug. Conventional separation techniques, such as
centrifugation, diafiltration, and molecular-sieve
chromatography are suitable. The composition can be
sterilized by filtration through a conventional 0.45
micron depth filter.
15

II. Utility

The significantly increased circulation half
life of liposomes constructed as above can be exploited
in two general types of therapeutic or diagnostic
20   liposome compositions. The first composition is
designed for sustained release of a liposome-associated
agent into the bloodstream by circulating liposomes. As
seen above, liposomes constructed according to the
invention can be maintained predominantly in the
25   bloodstream up to 24 hours, and therefore sustained
released of the drug at physiologically effective levels
for up to about 1 day or more can be achieved.

One measure of increased drug availability in
the bloodstream is the increased area under the curve
30   (AUC) seen with high blood/RES liposomes. The AUC mea-
surement is made, as described in Example 9, by measur-
ing liposome marker levels over a a 24 hour period, for
both blood and liver levels. The ratio of these AUC
values then shows the extent to total liposome

availability has been shifted from the liver to the
bloodstream. Table 7 in Example 7 demonstrates that
high blood/RES values are correlated with high AUC
ratios.

5   The extended lifetime of the liposomes in the
bloodstream also makes it possible for a significant
fraction of the injected liposomes to reach the target
site before being removed from the bloodstream by the
RES. In particular, it is desired to target tumor tis-
10   sue for drug treatment by intravenous administration to
a tumor-bearing subject.

          The use of the liposome composition of the
invention for targeting animal tumors is detailed in
Examples 11-13. Briefly summarizing the results,
15   liposomes with increased blood/RES ratios produced a
10-30 fold enhancement in tumor uptake over free drug.
Tumor uptake peaked at 24 hours post administration,
although high levels of drug in the tumor were seen
between 4 and 48 hours post administrations. Details of
20   the treatment methods are given in the examples.

          A variety of drugs or other pharmacologically
active agents are suitable for delivery by the liposome
composition. One general class of drugs include water-
soluble, liposome-permeable compounds which are charac-
25   terized by a tendency to partition preferentially into
the aqueous compartments of the liposome suspension, and
to equilibrate, over time, between the inner liposomal
spaces and outer bulk phase of the suspension. Repre-
sentative drugs in this class include terbutaline,
30   albuterol, atropine methyl nitrate, cromolyn sodium,
propranalol, flunoisolide, ibuprofin, gentamycin,
tobermycin, pentamidine, penicillin, theophylline,
bleomycin, etoposide, captoprel, n-acetyl cysteine,
verapamil, vitamins, and radio-opaque and

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particle-emitter agents, such as chelated metals.
Because of the tendency of these agents to equilibrate
with the aqueous composition of the medium, it is pre-
ferred to store the liposome composition in lyophilized
form, with rehydration shortly before administration.
Alternatively, the composition may be prepared in con-
centrated form, and diluted shortly before administra-
tion. The latter approach is detailed in US. Patent
application for "Liposome Concentrate and Method",
Serial No. 860,528, filed May 7, 1986.

A second general class of drugs are those
which are water-soluble, but liposome-impermeable. For
the most part, these are peptide or protein molecules,
such as peptide hormones, enzymes, enzyme inhibitors,
apolipoproteins, and higher molecular weight
carbohydrates are characterized by long-term stability
of·encapsulation. Representative compounds in this
class include calcitonin, atriopeptin, *a*-1_antitrypsin
(protease inhibitor), interferon, oxytocin, vasopressin,
insulin, interleukin-2, superoxide dismutase, tissue
plasminogen activator (TPA), plasma factor 8, epidermal
growth factor, tumor necrosis factor, lung surfactant
protein, interferon, lipocortin, *a*-interferon and
erythropoetin.

A third class of drugs are lipophilic mole-
cules which tend to partition into the lipid bilayer
phase of the liposomes, and which are therefore associ-
ated with the liposomes predominantly in a
membrane-entrapped form. The drugs in this class are
defined by an oil/water partition coefficient, as mea-
sured in a standard oil/water mixture such as
octanol/water, of greater than 1 and preferably greater
than about 5. Representative drugs include
prostaglandins, amphotericin B, progesterone, isosorbide

dinitrate, testosterone, nitroglycerin, estradiol,
doxorubicin, beclomethasone and esters, vitamin E, cor-
tisone, dexamethasone and esters, and betamethasone
valerete.

5   For sustained drug-release via the
bloodstream, the liposome composition is administered
intravenously in an amount which provides a suitable
drug dosage over the expected delivery time, typically
12-24 hours. The injection may be given as a single
10   bolus or slowly by i.v. drip, to allow gradual dispersal
of the liposomes from the site of injection.

           Where it is desired to target the liposomes to
a selected non-RES tissue site, the liposomes are
preferably designed for surface recognition of target-
15   site molecules. For example, in the case of targeting
to a solid tumor, the liposomes may be prepared with
surface-bound tumor recognition molecules, such as anti-
bodies directed against tumor-specific antigens. Methods
for coupling molecules of this type are wellknown to
20   those in the field. These methods generally involve
incorporation into the liposomes of lipid components,
such as phosphatidylethanolamine, which can be activated
for attachment of surface agents, or derivatized
lipophilic compounds, such as lipidderivatized
25   bleomycin.

           In one particular liposome composition which
is useful for radioimaging of solid tumor regions, the
liposomes are prepared with encapsulated radio-opaque or
particle-emission metal, typically in a chelated form
30   which substantially prevents permeation through the
liposome bilayer, and carrying surface-bound bleomycin
molecules, for preferential liposome attachment to tumor
sites.

The following examples illustrate methods of preparing liposomes with enhanced circulation times, and for accessing circulation times _in vivo_ and _in vitro_. The examples are intended to illustrate specific
5   liposome compositions and methods of the invention, but are in no way intended to limit the scope thereof.

Ceramides (CER), cholesterol (CH), monogalactosyl-sterate sulfatides (SULF),
10   galactocerebrosides (GAL), glucocerebrosides (GLU), and lactosylceremide (LAC) were obtained from Sigma (St. Louis, MO). Sphingomyelin (SM), egg phosphatidyl-choline (lecithin) (PC), phosphatidylinositol (PI), hydrogenated phosphatidylinositol (HPI), phosphatidyl-
15   serine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylethanolamine (PE), dipalmitoyl-phosphatidyl glycerol (DPPG), dipalmitoyl PC (DPPC), dioleyl PC (DOPC) and distearoyl PC (DSPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Globosides
20   (GLOB), digalactosyl diglyceride (DGDG), monosialoganglioside (GM_1), ganglioside GM_2 (GM_2), ganglioside GM_3 (GM_3), trisialoganglioside (GT_{1b}), and disialoganglioside (GD_{1a}) were obtained from Supelco (Bellefonte, PA).
25   [^{125}I]-tyraminyl-inulin was made according to published procedures. $^{67}Gallium$-8-hydroxyquinoline was supplied by NEN Neoscan (Boston, MA); doxorubicin (adriamycin), from Adria (Columbus, OH), and bleomycin, from Bristol Myers (Syracuse, NY).
30

Example 1
Preparation of REVs, MLVs and SUVs

This example describes the preparation of reverse phase evaporation vesicles (REVs), multilamellar vesicles (MLVs) and small unilamellar vesicles (SUVs).

A.   Sized REVs

A total of 50 mg of the selected lipid components, in the mole ratios indicated in the examples below, were dissolved in 5 ml of diethyl ether. An aqueous buffer containing 13 mM phosphate, 140 mM NaCl, pH 7.4 was added to the organic solvent to a final volume of 6.5 ml, and the mixture was emulsified by sonication for 1 minute, maintaining the temperature of the solution at or below room temperature. Where the liposomes were prepared to contain encapsulated $[^{125}I]$ tyraminyl-inulin, such was included in the phosphate buffer at a concentration of about 4 µCi/ml buffer.

The ether solvent was removed under reduced pressure at room temperature, and the resulting gel was taken up in 1 ml of the above buffer, and shaken vigorously. The resulting REV suspension had particle sizes, as determined by microscopic examination, of between about 0.1 to 20 microns, and was composed predominantly of relatively large (greater than 1 micron) vesicles having one or only a few bilayer lamellae.

The liposomes were extruded twice through a polycarbonate filter (Szoka, 1978), having a selected pore size of 0.4 microns or 0.2 microns. Liposomes extruded through the 0.4 micron filter averaged 0.17 (0.05) micron diameters, and through the 0.2 micron filter, 0.16 (0.05) micron diameters. Non-encapsulated $[^{125}I]$ tyraminyl-inulin was removed by passing the extruded liposomes through Sephadex G-50 (Pharmacia).

B.   MLVs

A total of 10-100 mg of the selected lipid
components were dissolved in chloroform: methanol (2:1).
5   The dissolved lipid was roto-evaporated to a thin film,
then hydrated with an aqueous physiological buffer con-
taining the solute, e.g., desferal or bleomycin, to be
encapsulated. MLVs formed on gentle shaking for 1-2
hours. Non-encapsulated solute was removed by gel fil-
10 tration. Examination of the MLVs showed heterogeneous
sizes between about 0.1 to 20 microns, and a predomi-
nance of multilayered structures. The MLV's were sized
by extrusion through a double polycarbonate membrane
having a selected pore size. The membrane pore size was
15 typically 0.05 micron for low phase transition (fluid)
liposome formulations, and 0.08 micron for high phase
transition (rigid) liposome formulations. In both
cases, liposome sizes, as measured by laser light scat-
tering, were predominantly in the 0.07-0.12 micron size
20 range. When high phase transition lipids were used,
rehydration and extrusion were carried at at 50°-60° C.

C.   SUVs

About 25 ml of the unsized MLV suspension from
25 above was sonicated by ultrasonic irradiation using a
1/2 inch saphire-bonded probe, with pulse sonication
during 15 minute intervals, under optimal output condi-
tions. Sonication was carried out under a stream of
nitrogen with the liposome vessel immersed in ice water.
30 The sonicated vesicles were passed through Sephadex G-50
to remove released, free marker. Liposome sizes were
predominantly in the 0.03-0.08 micron size range.

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Example 2
Measuring Blood/RES Levels

Age- and sex-matched mice, typically 20-25 g,
were given intravenous injections of liposome suspen-
sions or saline by injection into the tail vein. The
total amount of material injected was about 0.5 mg
phospholipid in a total injection volume of 0.2 ml. At
selected time intervals following injection, the animals
were sacrificed by cervical dislocation, blood samples
were taken from the heart, and the liver and spleen were
removed. The liver and spleen were blot dried, weighed
and separately counted directly by gamma scintillation
counting. A correction factor was applied to account
for blood remaining in the liver and spleen. An aliquot
of the blood sample was similarly counted by direct
gamma counting. Total blood counts were calculated on
the basis of a total blood volume of 7% body weight.
The blood/RES ratio was calculated as total blood
counts/total counts of the liver and spleen, and was
frequently determined for several other tissues.

The blood/RES ratios measured over time were
corrected for loss of liposomal radiolabel by multiply-
ing the measured blood/RES ratio by the percent of the
total counts remaining _in vivo_ at each time point with
respect to liposomal counts measured immediately after
injection.

Example 3
Relationship Between Lipid Composition and Blood/RES

REVs sized to 0.17 microns and having the
liposome composition and mole ratios indicated at the
left in Table 1 were prepared as in Example 1. The SM
used for the reported studies was bovine brain
sphingomyelin, whose hydrocarbon-chain moieties include

a mixture of partially unsaturated chains. The
liposomes were injected IV, and the injected animals
were sacrificed two hours after injection. The table
shows liposome sizes, blood/RES ratios, calculated as
above, and percent of total of the total encapsulated
marker (inulin) recovered at 2 hours post injection.

Table 1

	Composition	Size (μm)	Blood/RES	% remaining in vivo
1	PC	0.17	0.010 ± 0.005	86.0 ± 5.4
2	PC:CH,2:1	0.17	0.13 ± 0.08	78.1 ± 0.04
3	PC:GM_1,1:0.07	0.17	0.17 ± 0.12	79.4 ± 5.9
4	PC:CH:GM_1,2:1:0.14	0.16	1.7 ± 0.5	75.6 ± 5.7
5	PC:CH:$ASGM_1$,2:1:0.14	0.16	0.62 ± 0.44	64.8 ± 1.6
6	DSPC	0.17	0.015 ± 0.002	91.2 ± 2.00
7	DSPC:CH,2:1	0.17	0.007 ± 0.00	101.2 ± 2.4
8	DSPC:GM_1,1:0.07	0.17	2.0 ± 0.02	76.7 ± 3.1
9	DSPC:CH:GM_1,2:1:0.14	0.17	3.2 ± 1.0	64.6 ± 3.5
10	SM	0.17	0.02 ± 0.01	27.1 ± 3.1
11	SM:CH,2:1	0.17	0.7 ± 0.2	71.9 ± 4.4
12	SM:GM_1,1:0.07	0.17	5.7 ± 1.8	12.4 ± 0.7
13	SM:CH:GM_1,2:1:0.14	0.17	4.6 ± 0.6	72.1 ± 1.5
14	SM:PC,4:1	0.17	0.6 ± 0.2	69.0 ± 3.3
15	SM:PC:CH,4:1:3	0.17	0.12 ± 0.06	69.9 ± 2.2
16	SM:PC:CH:SULF,4:1:3:0.35	0.17	0.43 ± 0.21	78.4 ± 1.4
17	SM:PC:GM_1,4:1:0.35	0.16	3.3 ± 0.3	61.8 ± 2.9
18	SM:PC:CH:GM_1,4:1:3:0.35	0.16	1.5 ± 0.6	88.5 ± 3.0
19	SM:PC:$ASGM_1$,4:1:0.35	0.16	0.9 ± 0.5	80.3 ± 2.5

The blood/RES ratio data indicated that:
        1. Ganglioside GM_1 in liposomes also contain-
ing a membrane-rigidifying component, such as

cholesterol (composition 4), DSPC (composition 8), and
SM (compositions 12, 17), or combinations of these lipid
components (compositions 9 and 13) gave much higher
blood/RES ratios than liposomes containing membrane

5   rigidying components without GM_1 (compositions 6, 7, 10,
11, 14, or 15), or liposomes containing GM_1 but without
membrane rigidifying components (composition 3).
        2.   Substituting asilaoganglioside ($ASGM_1$) for
GM_1 in liposomes (composition 19 versus composition 17)

10   largely abolished the GM_1 enhancement effect on
blood/RES ratios.
        The percent total recovery data show two SM
formulations (compositions 10 and 12) which have rela-
tively poor persistence in the body, presumably because

15   of poor liposome stability and rapid clearance of the
released encapsulated marker.

                        Example 4
20   Effect of DSPC on Blood/RES
        Sized REVs having the five lipid compositions
shown at the left in Table 2, were prepared as above.
The compositions are similar to several of the Table 1
compositions, except that disteroyl PC (DSPC) has been

25   substituted for the relatively more unsaturated egg PC
(PC).   Blood/RES ratios were determined two hours after
IV administration as in Example 3, with the results
shown at the right in Table 2.   A comparison of
blood/RES vaues for comparable compositions in Tables 1

30   and 2 indicates that:
        1.   Substituting DSPC for PC in an SM:PC:GM_1
composition (composition 2 in Table 2 versus composi-
tion 17 in Table 1) significantly enhances the blood/RES
ratio.

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2.   Substituting DSPC for PC in the
SM:PC:CH:GM$_1$ composition (composition 5 in Table 2 ver-
sus composition 18 in Table 1) also enhances the
blood/RES ratio.

5

Table 2

Liposome composition	Blood/RES ratio
DSPC:GM$_1$, 5:0.35	1.8
SM:DSPC:GM$_1$, 4:1:0.35	4.9
SM:DSPC:GM$_1$, 1:1:0.14	3.7
SM:DSPC:GM$_1$, 1:4:0.35	3.1
SM:DSPC:CH:GM$_1$, 4:1:3:0.35	3.7

10

15   Example 5

Time Course of Blood/RES over 24 Hours

Blood/RES ratios for two liposome compositions
Sized (0.17 micron) REVs containing
(1) PC:CH:GM$_1$, 2:1:0.14 (composition 4), and (2)
20   SM:PC:GM$_1$, 4:1:0.35 (composition 17) were examined for
blood/RES values at 2, 6 and 24 hours post injection.
The behavior of the two formulations over a 24 hour
period is shown in Figure 1 for composition 4 (solid
circles) and composition 17 (open circles).

25   As seen, composition 1 was substantially com-
pletely removed after 2 hours, whereas composition 2
retains a significant level in the blood even at 24
hours post injection.

30   Example 6

Effect of Ganglioside Concentration on Blood/RES

Sized MLVs (0.17 microns) containing encapsu-
lated inulin were prwpared as above. The liposome com-
positions contained either PC:CH, 2:1 (Figure 2A), or

SM:PC, 4:1 (Figure 2B) and increasing molar amounts of GM_1, including 0, 2.5, 5.0, 7.5, and 10, 12 and 15 mole percent. The tissue distribution of the liposome label in liver (triangles), spleen (squares), and blood (cir-
5   cles) were determined, as above, two hours post IV administration.

       With reference to to Figure 2A, increasing molar amounts GM_1 increased blood levels two hours post injection to at most about 10% of total injected counts
10   in liposomes containing 5 and 7.5 mole percent ganglioside. At the same time, the lowest levels of liver uptake which were observed, also at 5 and 7.5 mole percent ganglioside, were greater than 30% of total injected counts.

15   By contrast, with reference to Figure 2B, increasing amounts of GM_1 above about 2.5 mole percent increased the percent counts in the blood to above 50%, while total liver uptake fell to less than about 10%. The results demonstrate the importance of
20   membrane-rigidifying components--in this case, SM-- to the effect of GM_1 on blood/RES ratios. The results also show that optimal GM_1 concentrations of in the liposomes are between about 7.5 and 15.0 mole percent.

25   Example 7
Effect of Sugar and Negatively Charged Groups on Blood/RES

       Sized REVs having one of the 18 different com-
positions shown in Table 3 were prepared, to determine
30   the effect of various sugar and/or negatively charged groups on blood/RES levels 2 hours after liposome admin-
istration. Each of the compositions contained PC:CH, 2:1 and 0.2 mole percent of one or more glycolipid or negatively charged lipid. The various charged and/or

glycolipids used were: monogalactosyl-stearate,
sulfatide (SULF) having a charged sulfate group on the
galactose residue (Figure 5c); phosphatidylserine (PS);
phosphatidic acid (PA), ceramide trihexosides, (CTRI),
5   an uncharged glycolipid with three hexose units;
digalactosyl diglyceride (DGDG), an uncharged glycolipid
with two galactosyl residues; monogalactosyl
diglyceride (MGDG), an uncharged glycolipid with a sin-
gle galactosyl residue; galactocerebrosides (GAL), an
10   uncharged glycolipid with a single galactosyl residue;
globosides (GLOB), an uncharged glycolipid with 4 sugar
residues; asialoganglioside (ASGM$_1$); and
glucocerebroside (GLU), an uncharged glycolipid with a
single glucose residue.

15   The molar compositions, and blood/RES ratios
measured for 2 hours post injection are given in Table 3
below. In the compositions containing PC and CH, only
GM$_1$ or GAL or GLU in combination with GM$_1$ gave blood/RES
values which were significantly greater than that given
20   by PC:CH liposomes. PS and PA both decreased blood/RES
ratios, with PS producing an extreme reduction in
blood/RES.

         In the compositions containing SM and PC,
monogalactosyl-stearate (SULF) gave values comparable to
25   the PC:CH:GM$_1$ formulations.

30

Table 3

Lipid composition*	size	blood/RES, 2 hrs post injection	% remaining in vivo
PC:CH, 2:1	0.1 μ	0.68±0.15	53.3±6.6
PC:CH:SULF, 2:12:0.2	0.1 μ	0.54±0.22	63.3±1.3
PC:CH:PS, 2:1:0.2	0.1 μ	0.02±0.00	79.2±7.2
PC:CH:PA, 2:1:0.2	0.1 μ	0.09±0.01	62.6±2.2
PC:CH:CTRI, 2:1:0.2	0.1 μ	0.15±0.02	81.7±2.3
PC:CH:DGDG, 2:1:0.2	0.1 μ	0.74±0.44	43.2±7.2
PC:CH:MGDG, 2:1:0.2	0.1 μ	0.31±0.08	67.4±3.2
PC:CH:GAL, 2:1:0.2	0.1 μ	1.01±0.63	8.7±0.2
PC:CH:GLOB, 2:1:02	0.1 μ	0.08±0.03	74.2±2.1
PC:CH:ASG$_{M1}$, 2:1:0.2	0.1 μ	0.46±0.01	75.2±8.1
PC:CH:GLU, 2:1:0.2	0.1 μ	0.72±0.21	84.6±12.1
PC:CH:G$_{M1}$, 2:1:0.2	0.1 μ	4.3±1.1	75.9±3.1
PC:CH:GAL:GM$_1$, 2:1:0.2	0.1 μ	5.95±1.42	56.8±5.0
PC:CH:GLU:GM$_1$, 2:1:0.2	0.1 μ	3.98±0.91	81.3±3.3
SM:PC, 1:1	0.2 μ	2.14±1.36	72.05±5.57
SM:PC:SULF, 1:1:0.2	0.2 μ	3.08±3.41	82.4±10.8
SM:PC:PA, 1:1:0.2	0.2 μ	1.20±0.31	74.4±2.9
SM:PC:PS, 1:1:0.2	0.2 μ	0.02±0.00	81.3±1.8

Blood/RES values were measured at 2 hours post
injection for sized REVs (0.17 micron) composed of
SM:PC, 4:1 and 0.35 mole percent of one of the following
gangliosides or modified gangliosides: GM$_1$; GM$_2$, con-
taining one less uncharged terminal sugar residue; GM$_3$,
containing two less uncharged sugar residues;
disialoganglioside (GD$_{1a}$), whose four sugar residues
contain two sialic acid moieties; and
trisialoganglioside (GT$_{1b}$), whose four sugar residues
contain two sialic acid moieties. The blood/RES ratios
are shown in Table 4 below. As seen, only the GM$_1$
ganglioside gives high blood/RES ratios.

Table 4

Lipid composition	Blood/Res
SM:PC:GM$_1$	3.3 (0.3)
SM:PC:GM$_2$	0.6 (0.3)
SM:PC:GM$_3$	0.3 (0.2)
SM:PC:GD$_{1a}$	0.6 (0.3)
SM:PC:GT$_{1b}$	0.3 (0.3)
SM:PC:ASGM$_1$	0.9 (0.5)

Example 8

Liposome Distribution 4 Hours Post Injection

MLVs were prepared as in Example 1, using a
hydration buffer containing 25 mM desferal. The lipid
compositions are listed at the left in Table 5 below,
and the relative molar quantities in the adjacent col-
umn. The MLVs were extruded as in Example 1, to produce
a size range between about 0.07-1.12 microns, and free
desferal was removed by Sephadex™ G-75 column
chromatography.

One day before animal injection, a complex of
^{67}gallium/8-hydroxyquinoline (a weak lipophilic
chelator) was added to the liposome suspension. When
this complex penetrates the liposomes, ^{67}Ga transloca-
tion to encapsulated desferal occurs. The resulting
^{67}Ga-desferal complex has high affinity and is not dis-
placeable by transferrin or other metal-binding pro-
teins. If released from liposomes, the complex is rap-
idly excreted through the urine with a half life of a
few minutes. Immediately before injection, the
liposomes are passed through an anion exchange resin
(AG-1x4 acetate form) which compleletly removes all non-
encapsulated ^{67}Ga-8-hydroxyquinoline complex.

Age- and sex-matched mice were injected intra-
venously through the tail vein with a liposome prepara-
tion having one of the lipid compositions shown in Table
5 below. Four hours after injection, the levels of
5   radioactivity (counts per minute) in blood and dissected
body parts, including liver and spleen were measured by
gamma counting, using integral counting between 10 and
1,000 kev. Total blood counts were determined as above,
based on an estimated total blood volume. Percent total
10   recovery was determined by whole body gamma counts
(whole body counts x 100/injected counts).

The blood/RES ratio was calculated as above,
by dividing total blood counts by the sum of total liver
and spleen counts, with the results shown in Table 5.
15   As seen, both GM_1 ganglioside and hydrogenated PI (HPI)
gave relatively high blood/RES ratios in liposomes con-
taining saturated PC or saturated PC plus SM. The same
ganglioside or SULF in an unsaturated liposome formula-
tion, or egg PI in an unsaturated formulation gave sub-
20   stantially lower values.

25

30

Table 5

	Liposome Composition	Molar Ratio of Components	Blood/RES Ratio	% Total Recovery
5	PG:PC:CH	1:10:5	.083	76.1 (0.8)
	PG:PC:CH (unextruded)	1:10:5	.006	55.8 (2.9)
	GM_1:PC:CH	1:10:5	1.1	63.2 (2.0)
	SULF:PC:CH	1:10:5	1.1	61.0 (0.9)
10	DPPG:DSPC:CH	1:10:5	2.0	88.6 (6.1)
	PI:PC:CH	1:10:5	.83	49.0 (8.3)
	HPI:DSPC:CH	1:10:5	2.5	78.7 (1.5)
	GM_1:SM:DSPC:CH	1:8:2:5	5	50.3 (3.4)
	GM_1:DSPC:CH	1:10:5	5	88.5 (1.2)
15	GL_4:PC:CH	1:10:5	.02	--
	GT_1:PC:CH	1:10:5	.185	--

Example 9

Liposome Distribution 24 hours Post Injection

20   Sized MLVs having the lipid compositions shown
in Table 6 and encapsulating ^{67}gallium/desferal complex
were prepared as in Example 8. Blood/RES ratios and
total percent body recovery, determined as above, are
shown at the right in the table. The data show that
25 GM_1, HPI and SULF in combination with saturated neutral
lipid components, such as DSPC and SM, give optimal
blood/RES ratios at 24 hours.

        Lower blood/RES values were obtained where the
negatively charged component is PG or DSPG, in which the
30 negative charge is shielded by the relatively small
glycerol moiety; and cholesterol sulfate (CHS), in which
the charged sulfate group is exposed.

Table 6

	Liposome Composition	Molar Ratio of Components	Blood/RES Ratio	% Total Recovery
5	GT_1:PC:CH	1:10:5	0.004	58.2 (8.8)
	PG:PC:CH			
	(unextruded)	1:10:5	0.004	31.3 (1.5)
	GLOB:PC:CH	1:10:5	.008	34.3 (9.2)
	PG:PC:CH	1:10:5	.008	49.8 (3.9)
10	DSPC:CH	10:5	.014	67.6 (4.2)
	SULF:PC:CH	1:10:5	.02	41.6 (2.5)
	SM:PC:CH	8:2:5	.03	21.9 (1.4)
	SM:PC	8:2	.03	6.3 (1.2)
	HPI:DOPC:CH	1:10:5	.06	46.3 (1.8)
15	CHS:DSPC:CH	1:10:5	.08	55.6 (6.0)
	GM_1:SM:PC	1:8:2	.1	15.1 (0.5)
	PC:CH	10:5	.11	44.4 (2.9)
	GM_1:SM:PC:CH	1:8:2:5	.12	14.4 (1.9)
	DPPG:DSPC:CH	1:10:5	.2	59.9 (5.5)
20	PG:DSPC:CH	1:10:5	.2	12.9 (1.4)
	PI:PC:CH	1:10:5	.28	37.4 (6.3)
	SULF:DSPC:CH	1:10:5	.3	57.5 (3.7)
	GM_1:PC:CH	1:10:5	.33	40.3 (1.5)
	HPI:DSPC:CH	1:10:5	.43	61.6 (4.0)
25	GM_1:SM:DSPC:CH	1:8:2:5	.5	36.9 (5.3)
	HPI:HPC:CH	1:10:5	.55	51.1 (6.7)
	GM_1:DSPC:CH	1:10:5	.9	66.3 (4.2)

Also of note are the varied total body recov-
30 eries after 24 hours with the different formulations.
Although there was no identifiable relationship between
percent body recovery to blood or RES values, or their
ratio, many of the formulations showed relatively low
recoveries where the lipid components had widely varying

phase transition temperatures (T_p). Thus, for example, the GM$_1$ formulations containing SM (T_p about 30°C) and DSPC (T_p about 50°C) gave much lower recovery than the formulation without SM, also as observed at 4 hours post injection. Even more striking was the low recovery seen for the ganglioside or non-ganglioside formulations containing SM and egg PC (T_p about 0°C), with or without cholesterol.

The total radioactivity levels (liposomal marker) contained in the blood, liver and spleen over a 24 hour post injection period were determined for several of the liposome compositions, shown at the right in Table 7 below. These levels were determined as area under the curve (AUC) for levels measured at 2, 4 and 24 hours, and thus are related to total levels of marker present in the tissue (blood, liver or spleen) in the period 24 hours post injection.

As seen from the table, high AUC blood/liver ratios were observed for the formulations containing GM$_1$ or HPI; and PG or DPPG formulations gave much lower ratios, consistent with the blood/RES ratios in Table 6.

Table 7

Liposome Composition	Molar Ratio of Components	Blood Liver (AUC)		Blood/ Liver AUC Ratio
PG:PC:CH	1:10:5	0.52	7.34	.07
DPPG:DSPC:CH	1:10:5	3.52	4.27	.82
GM$_1$:PC:CH	1:10:5	2.66	1.68	1.58
GM$_1$:SM:DSPC:CH	1:8:2:5	2.21	1.76	1.25
GM$_1$:DSPC:CH	1:10:5	5.84	1.95	3.0
HPI:DSPC:CH	1:10:5	3.82	3.31	1.15

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    To examine whether a statistically significant
correlation exists between liposome blood levels on one
hand, and RES levels on the other, a linear regression
analysis of the data, using the least squares method,
5   was performed. Figure 3A shows the inverse (negative)
correlation between blood levels and RES uptake
($r=-0.88$, $p=0.00002$). The values were taken from
Table 6.

10   Example 10
            Effect of Charge on Blood/RES Ratios
            Sized MLVs containing increasing molar amounts
of HPI or PI and either DSPC or PC plus cholesterol, as
indicated in Table 8 below, were prepared as above, and
15 examined for blood/RES ratios and total body recovery 24
or 4 hours post injection. As seen, increasing the
molar percentage of HPI from about 6 to 16 produced lit-
tle change in the blood/RES ratio, whereas the ratio
dropped dramatically between 16 and 30 percent. A simi-
20 lar drop in ratio was observed for the PI formulations,
both 4 and 24 hours post injection. The percent PI or
HPI had no major effect on total body recovery after 4
or 24 hours.

25

30

Table 8

Liposome Composition	Molar Ratio of Components	% PG	Blood/RES Ratio	% Total Recovery
24 Hours				
MPI:DSPC:CH	1:10:5	6.3	.55	62.2 (6.0)
HPI:DSPC:CH	2.5:7.5:5	16.7	.45	59.9 (3.5)
HPI:DSPC:CH	4.5:5.5:5	30.0	.008	48.3 (1.5)
PI:PC:CH	1:10:5	6.3	.27	37.4 (6.3)
PI:PC:CH	4:6:5	26.7	.03	53.4 (15.3)
4 Hours				
PI:PC:CH	1:10:5	6.3	.83	49.0 (8.3)
PI:PC:CH	4:6:5	26.7	.07	72.4 (2.3)

Example 11
Uptake of Liposomal Marker into Mouse Tumors

Animals were inoculated with J6456 or B16 tumor cell lines. The J6456 line is a T-cell derived lymphoma (Gabizon) that will grow in vitro as a cell suspension, and after intraperitoneal injection, as an ascitic tumor. After IV injection, it will metastasize predominantly into the liver and spleen. The B16 melanoma line is a neuroectodermal-derived, solid type of tumor which grows as an adherent tumor in vitro, and metastasizes mainly in the lungs.

Tumor cells (10^6 J6456 or 5×10^5 B16 cells) were inoculated intramuscularly (IM) in the hind leg of syngeneic (C57BL/6 or Balb/c) femal mice. Between 2-3 weeks after inoculation, when tumors weighed approximately 0.5 to 2 g, mice were injected IV with 1 umol phospholipid of one of the liposome compositions shown in Table 9 below. The molar ratios of components is the same as for the same-lipid component formulations. The

liposomes were sized MLVs prepared as above with encap-
sulated gallium/desferal complex, and having sizes pre-
dominantly in the 0.07 to 0.12 micron size range.

Complete animal dissection followed 24 hours
after liposome administration. The values shown in the
table are based on [67]gallium counts and corrected for
blood content of the tissues. The correction factor for
blood content in normal tissues and tumor was determined
by examining the distribution of ^{111}In-oxine-labeled red
blood cells in age- and sex-matched tumor-bearing mice.
Ratios were obtained by dividing the percents of
injected dose per gram of respective tissues. Body
average represents the average liposome uptake per gram
body weight, and was calculated by dividing the percent
of injected dose recovered in the total body (including
tumor) by the weight of the animal.

As seen from the data in Table 9, for the
J6456-injected mice, a steady increase in tumor uptake
(up to 25 fold) was observed with liposomes selected for
longer _in vivo_ circulation times. Values of between
about 4-6% of the injected dose were obtained with the
formulations containing GM$_1$ ganglioside or with HPI in
combination with saturated phospholipids. These values
were obtained after correcting for blood volume in the
tumor, as above. When free [67]gallium-desferal complex
was injected, the tumor uptake of the marker was less
than 0.1% of the injected dose per gram. The data at
the right in the table show a concomitant decrease in
liver-to-tumor ratios, indicating that liposomes accumu-
late preferentially in tumors as opposed to non-specific
enhancement in all body tissues. Progressively higher
tumor-to-carcass ratio were also seen with the GM$_1$ or
HPI formulations (data not shown).

Table 9

Liposome Composition	Percent of Injected Dose/G (SD)			Ratio: Tumor/ Body Avg	Ratio: Liver/ Tumor
	Tumor	Liver	Body Avg		
PG:PC:CH	0.2 (0.0)	36.4 (6.3)	2.9 (0.7)	0.1	182.0
PG:PC:CH (unextruded)	0.3	21.4	2.3	0.1	71.3
SULF:PC:CH	0.8	13.6	2.0	0.4	17.0
DSPC:CH	2.1 (0.3)	36.5 (7.5)	2.8 (0.3)	0.8	17.4
SULF:DSPC:CH	2.1 (0.3)	32.1 (4.5)	2.1 (0.1)	1.0	15.3
CHS:DSPC:CH	2.5 (0.1)	29.7 (1.4)	2.3 (0.2)	1.1	11.9
HPI:DSPC:CH	4.1 (1.1)	37.8 (0.4)	3.0 (0.2)	1.4	9.2
DPPG:DSPC:CH	4.1 (1.6)	38.3 (0.5)	2.8 (0.1)	1.5	9.3
GM_1:DSPC:CH	5.3 (0.9)	31.7 (1.4)	3.3 (0.1)	1.6	6.0
GM_1:PC:CH	3.5 (0.6)	20.8 (0.9)	2.4 (0.2)	1.5	5.9

Similar conclusions are drawn from the tumor uptake data in animals inoculated with the B16, shown in Table 10, although tumor uptake increases as a function of liposome composition are less dramatic.

Table 10

Liposome Composition	Percent of Injected Dose/G (SD)			Ratio: Tumor/ Body	Ratio: Liver/ Tumor
	Tumor	Liver	Body		
GM$_1$:PC:CH	2.5 (0.7)	24.3 (1.3)	2.5 (0.1)	1.0	9.7
SULF:DSPC:CH	3.6 (0.8)	23.5 (1.4)	3.1 (0.3)	1.2	6.5
PG:DSPC:CH	1.5 (0.4)	9.8 (0.6)	1.2 (0.2)	1.3	6.5
DSPC:CH	5.4	33.2	4.2	1.3	6.1
CHS:DSPC:CH	5.7 (0.5)	21.1 (1.9)	3.5 (0.2)	1.6	3.7
DPPG:DSPC:CH	4.9 (0.3)	17.8 (2.2)	2.9 (0.1)	1.7	3.6
GM$_1$:DSPC:CH	8.4 (0.3)	37.2 (7.2)	4.4 (0.4)	1.9	4.4
HPI:DSPC:CH (1:10:5)	5.3 (0.3)	14.1 (0.3)	2.9 (0.1)	1.8	2.7
HPI:DSPC:CH (2.5:7.5:5)	5.2 (1.5)	19.7 (2.1)	3.3 (0.3)	1.6	3.8
HPI:DSPC:CH (4.5:5.5:5)	.3 (1)	44.1 (1.9)	2.9 (0.1)	0.1	147.0

Linear regression analysis of the data, using least squares analysis, was carried out to determine whether a statistically significant correlation exists between liposome blood levels and tumor uptake. Figure 3B shows a direct (positive) correlation between blood levels and tumor uptake of liposomes in both the B6456 (closed triangles) and B16 (closed circles) animals. The correlation coefficient for the J6456 animals are r=0.89 and p=0.0005, and for the B16 animals, r=0.91 and p=0.004.

Example 12
Uptake of Indium-Labeled Bleomycin into Mouse Tumors

Sized MLVs composed of GM$_1$:DSPC:CH (1:10:5) and containing encapsulated ^{111}In-bleomycin were prepared as above. Bleomycin was labelled with ^{111}In by adding the label to a suspension of bleomycin liposomes

one day before liposome administration, to form a
high-affinity, ^{111}In-bleomycin complex encapsulated in
the liposomes. Immediately before use the liposomes
were passed through an anion-exchange resin, as above,

5 to remove non-encapsulated ^{111}In.

Mice innoculated with J6456 lymphoma or B16
cells were injected IV with liposomes (1 umole
phospholpid/animal) or with an equivalent amount of free
^{111}In-bleomycin. Tissue distribution of the radiolabel,

10 24 hours post administration, was determined as above,
with the results shown in Tables 11 (J6456-infected ani-
mals) and Table 12 (B16-infected animals). As seen, the
liposomal form of the drug increased drug quantity 10-30
fold over free drug.

15

Table 11
Percent of Injected Dose /g Tissue

	TISSUE	FREE ^{111}In-BLEO	^{111}In-BLEO
20	TUMOR	0.7	8.2 (1.5)
	BLOOD	0.9	2.5 (0.9)
	LIVER	0.9	44.8 (0.6)
25	BODY AVG	0.5	4.3 (0.1)

30

Table 12

Percent of Injected Dose / Tissue

TISSUE	FREE ^{111}In-BLEO	Liposomal ^{111}In-BLEO
TUMOR	0.3 (0.0)	9.2 (1.9)
BLOOD	0.1 (0.0)	7.4 (0.8)
LIVER	0.4 (0.0)	23.0 (1.1)
BODY AVG	0.2 (0.0)	4.0 (0.3)

Sized HPI:DSPC:CH (1:10:5) liposomes with encapsulated ^{111}In-bleomycin were prepared as above and injected IV into mice inoculated with J6456 tumor cells, as above. Animals were sacrificed at 4, 24 and 48 hours and tumor, blood and liver levels of radioactivity determined, with the results shown in Table 13 below. Blood levels of the label dropped rapidly during the 4-48 hour test period. With both liver and tumor, optimal levels were observed at 24 hours, although both 4 and 48 hour levels were relatively high.

Table 13

Percent of Injected Dose / Tissue

Tissue	4 hours	24 hours	48 Hours
Blood	47.9 (2.5)	8 (1.5)	0.7 (0.1)
Liver	20.4 (1.6)	30.6 (1.4)	21.4 (0.8)
Tumor	7.8 (1.2)	13.7 (1.1)	10.5 (0.7)

Example 13

Uptake of Doxorubicin into J6456 Mouse Tumors

Sized MLVs having one of the three lipid compositions shown in Table 14 below were prepared as above. Doxorubicin was included in the hydration buffer, at a concentration of about 5 mg/ml, and free drug was removed from the sized liposomes by gel filtration.

Mice innoculated with J6456 lymphoma cells were injected IV with the MLVs (1 umole phospholpid/animal) or with an equivalent amount of free doxorubicin. Tissue distribution of the drug, 24 hours post administration, was determined fluorometrically, with the results shown in Tables 13 below. As seen, drug levels of the drug, expressed as percent of injected dose/g tumor, were similar to free drug for the two liposome compositions (PG:PC:CH and GM_{11}:PG:PC:CH) which do not show significantly enhanced blood/RES ratios, whereas the drug level in tumors was enhanced 3-6 fold with GM_1:DSPC:CH liposomes which show optional blood/RES ratios.

Table 14

Percent of Injected Dose (DXR)/ Tumor (SD)

FREE DXR	0.4 (0.1)
PG:PC:CH (DXR) (1:10:5)	0.2 (0.0)
GM_1:PG:PC:CH (DXR) (1:10:5)	0.2 (0.0)
GM_1:DSPC:CH (DXR) (1:10:5)	1.3 (0.1)

While specific methods of preparing and using the liposomes of the invention have been illustrated herein, it will be apparent that a variety of different

lipid compositions, drug-liposome formulations, and liposome treatment methods can be practiced within the scope of the invention.

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IT IS CLAIMED:

1. A composition comprising liposomes which contain an entrapped pharmaceutical agent and are char-

5 acterized by:

(a) liposome sizes predominantly between about 0.05 and 0.5 microns,

(b) liposomes having substantially homogeneous-phase bilayers composed of at least about 50

10 mole percent of a membrane-rigidifying lipid selected from the group consisting of sphingomyelin and neutral phospholipids with predominantly saturated acyl chains, and

(c) between about 5-20 mole percent of a

15 glycolipid selected from the group consisting of ganglioside GM_1, saturated phosphatidylinositol, and galactocerebroside sulfate ester.

2. The composition of claim 1, which is char-

20 acterized by a tissue distribution ratio, as measured by the amount of intravenously administered liposomes in the blood divided by the combined amount of administered liposomes in the liver and spleen, when measured 2 hours after intravenous administration of the composition to a

25 subject, which is substantially greater than the sum of the tissue distribution ratios obtained with similarly constructed liposome compositions containing in one case, at least about 50 mole percent of the membrane rigidifying lipid, but not the glycolipid, and in the

30 other case, between 5-20 mole percent of the glycolipid, but not the not the membranerigidifying lipid.

3. The composition of claim 1, wherein the glycolipid is ganglioside GM_1.

        4.   The composition of claim 1, wherein the glycolipid is hydrogenated phosphatidyinositol.

5   5.   The composition of claim 1, wherein the liposomes are predominantly in the 0.07 to 0.02 micron size range.

        6.   The composition of claim 1, wherein the
10   membrane-rigidifying lipid is brain sphingomyelin liposomes contain sphingomyelin and the liposomes contain sphingomyelin and phosphatidylcholine, at a mole ratio between 2:1 and 4:1

15   7.   The composition of claim 1, wherein the membrane-rigidifying lipid is predominantly phosphatidylcholine with saturated acyl chains.

        8.   In a therapeutic drug treatment in which a
20   drug is administered intravenously in a suspension of liposomes in which the drug is entrapped and whose sizes are predominantly between about 0.05-0.5 microns, a method of extending the lifetime of liposomes in the bloodstream which comprises preparing the liposomes to
25   contain:

        (a) substantially homogeneous-phase bilayers containing at least about 50 mole percent of a membrane-rigidifying lipid selected from the group consisting of sphingomyelin and neutral phospholipids with
30   predominantly saturated acyl chains, and

        (b) between about 5-20 mole percent of a glycolipid selected from the group consisting of ganglioside GM_1, saturated phosphatidylinositol, and galactocerebroside sulfate ester.

9.   The method of claim 8, wherein the tissue
distribution ratio produced by injecting the liposomes
intravenously, as measured by the amount of liposomes in
5   the blood divided by the combined amount of administered
liposomes in the liver and spleen, when measure 2 hours
after intravenous administration of the composition to a
subject is substantially greater than the sum of the
tissue distribution ratios obtained with similarly con-
10   structed liposome compositions containing in one case,
at least about 50 mole percent of the membrane rigidify-
ing lipid, but not the glycolipid, and in the other
case, between 5-20 mole percent of the glycolipid, but
not the not the membranerigidifying lipid.
15

10.   The method of claim 8,   wherein the
glycolipid is ganglioside GM_1.

11.   The method of claim 8 wherein the
20   glycolipid is hydrogenated phosphatidylinositol.

12.   The method of claim 8, wherein the
liposomes contain sphingomyelin and phosphatidylcholine,
at a mole ratio between 2:1 and 4:1.
25

13.   The method of claim 8, wherein the
membrane-rigidifying lipid is predominantly
phosphatidylcholine with saturated acyl chains.

30   14.   The method of claim 8, for use in tumor
therapeutic drug treatment, wherein the amount of drug
which is delivered to the tumor, as measured by the
amount drug/tumor weight 24 hours after drug

administration, is severalfold greater than that achievable by administering the drug in free form.

5   15.   The method of claim 14, wherein drug which is entrapped in the liposomes is bleomycin.

16.   The method of claim 14, wherein the drug which is entrapped in the liposomes is doxorubicin.

10   17.   The method of claim 14, which further includes targeting the liposomes to the tumor by addition of tumor-specific binding molecules to the liposome surfaces.
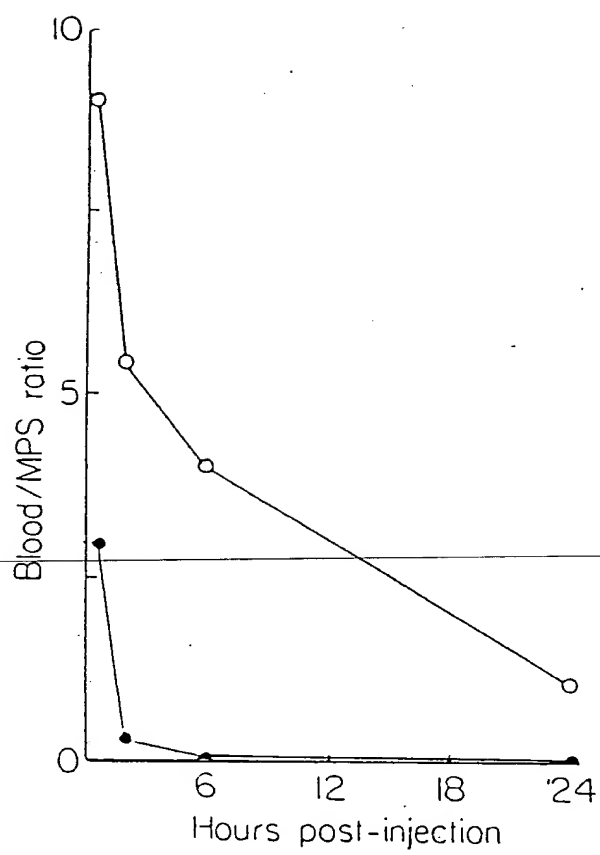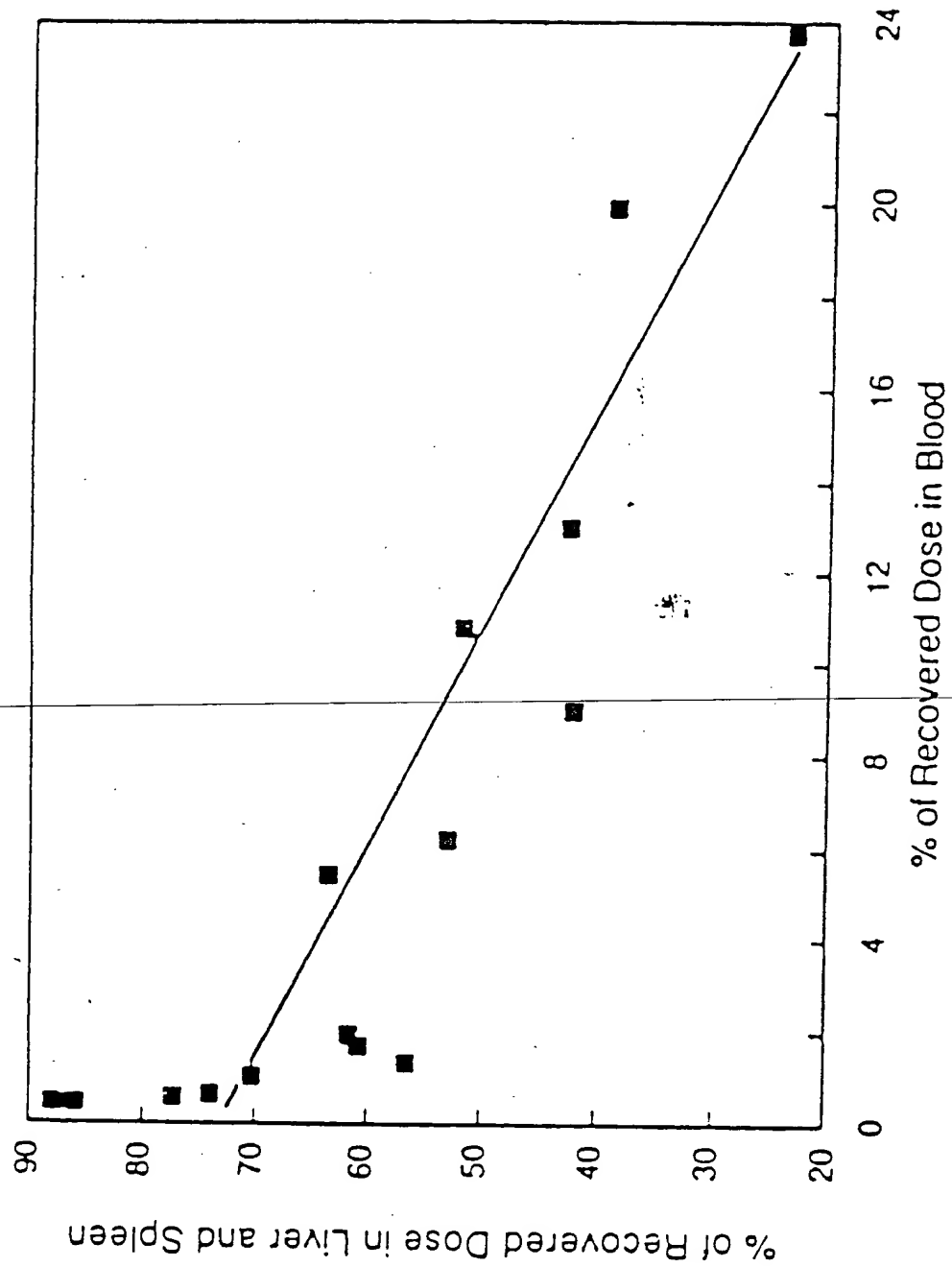
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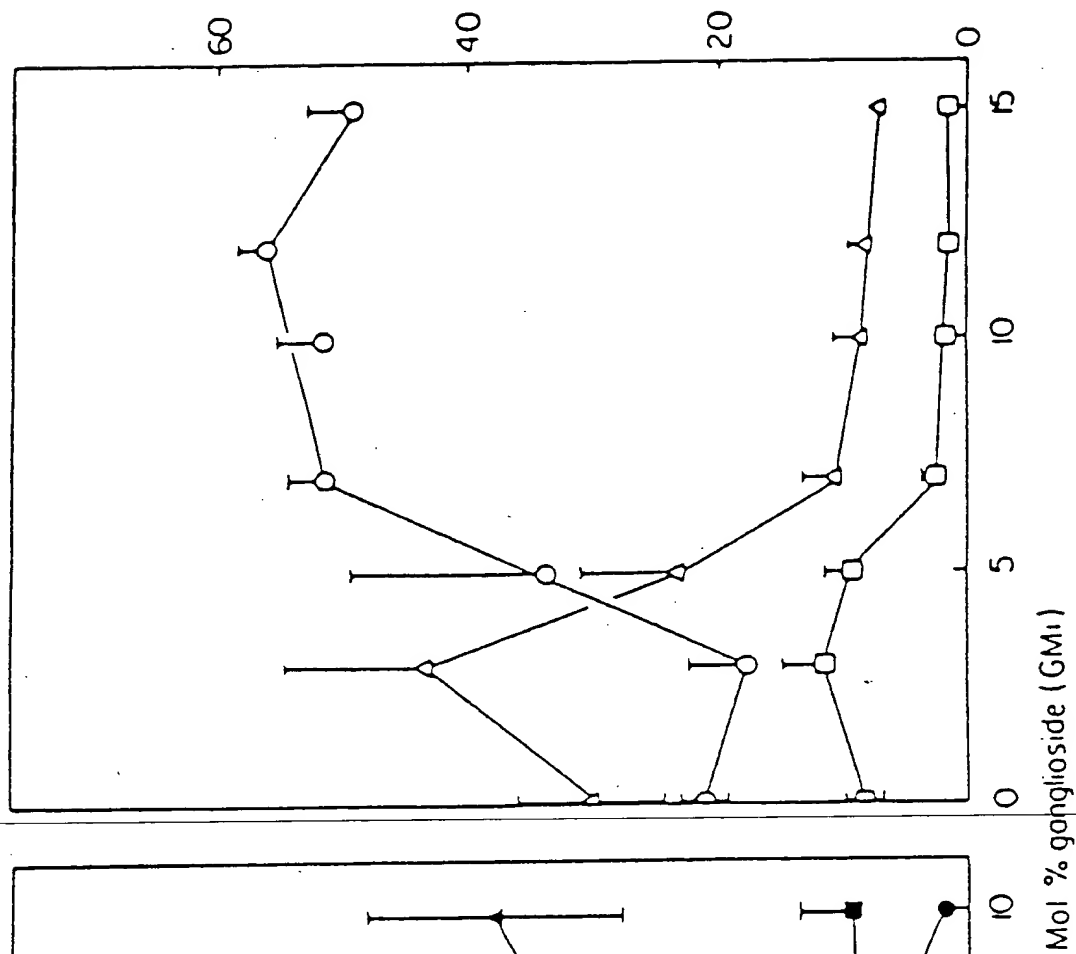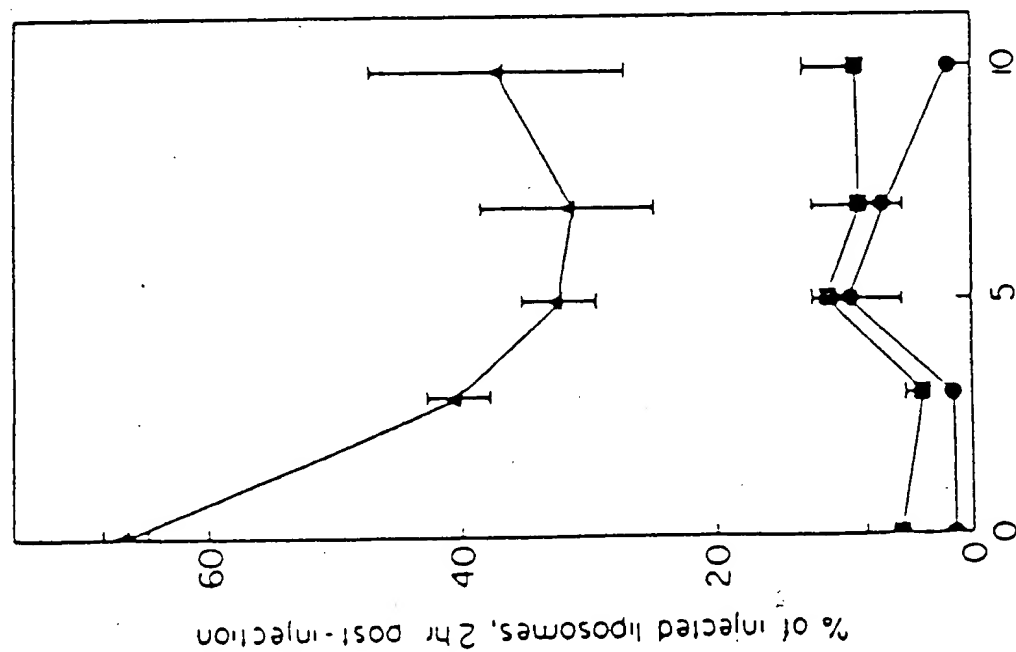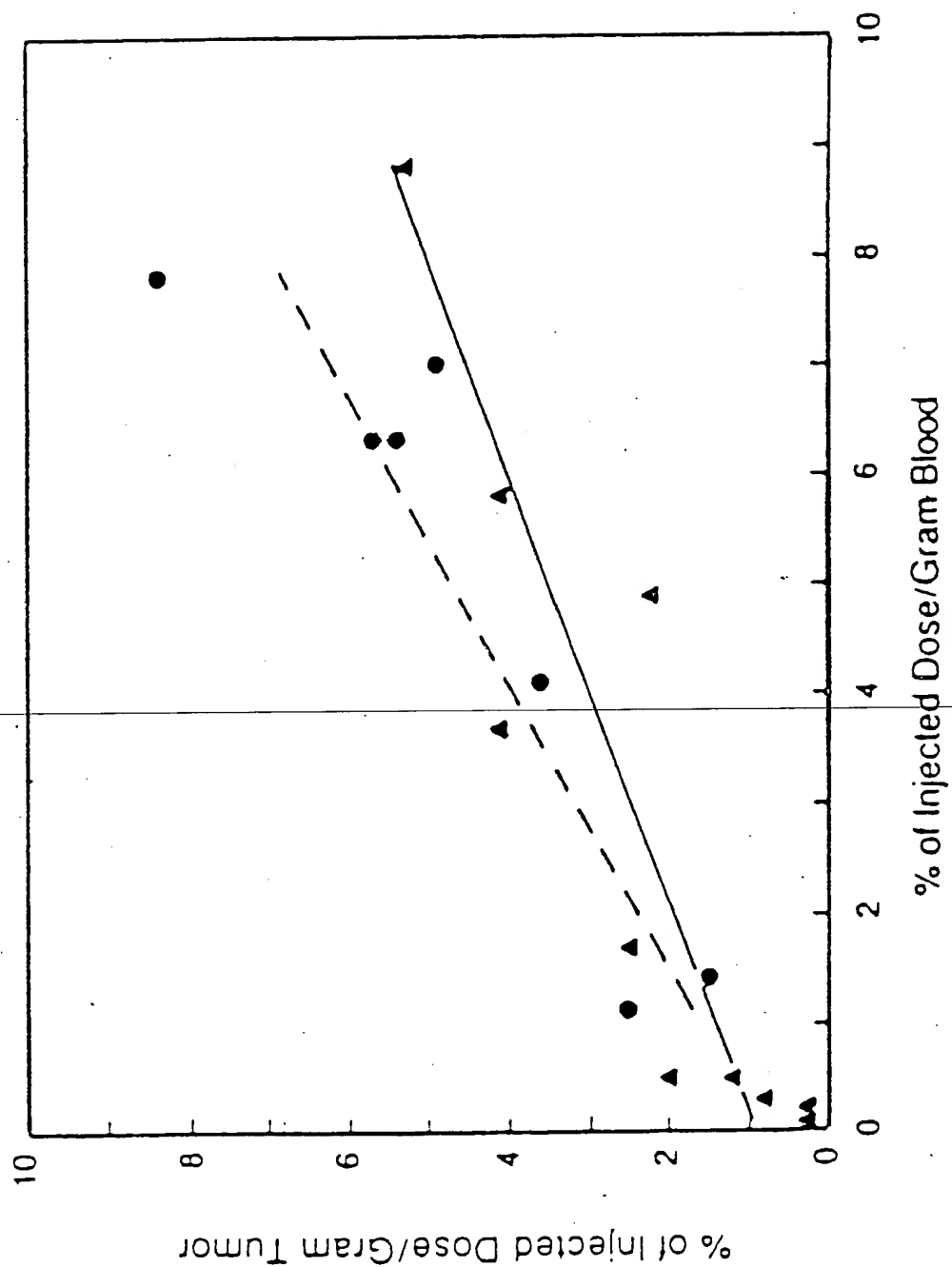
FIG. 1

FIG. 2B

FIG. 2A

Mol % ganglioside (GM₁)

% of injected liposomes, 2hr post-injection

FIG. 3A

% of Recovered Dose in Liver and Spleen

% of Recovered Dose in Blood

FIG. 3B

% of Injected Dose/Gram Blood

% of Injected Dose/Gram Tumor

FIG. 4A

FIG. 4B

FIG. 4C

SULF
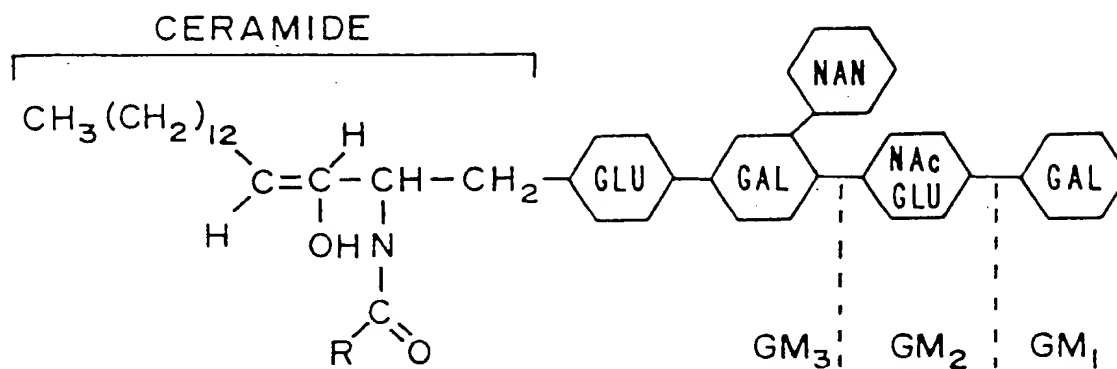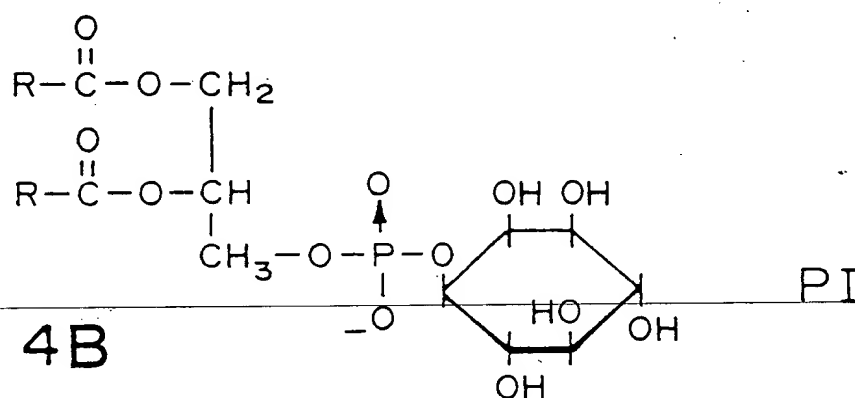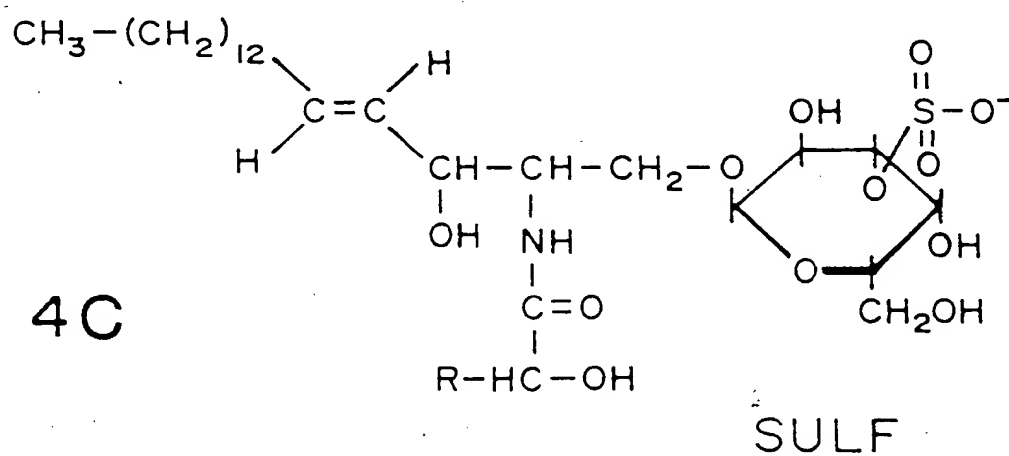
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. Cl. 4 A61K9/42,9/52; B01J 13/02; C07F 9/10
U.S. Cl. 260/403; 424/450; 428/402.2; 436/829

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S. Cl.	260/403; 424/450; 428/402.2; 436/829

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X Y	US, A, 4,598,051 Published 01 July 1986 PAPAHADJO-POULOS et al. See Col. 4, line 17–col. 6, line 3; Col. 11, lines 11–46 and 61–68; col. 14, lines 1–51; col. 15, lines 51–55; and col. 1, lines 20–29.	1–14,17 4,11,15,16
Y	US, A, 2,870,179 Published 20 January 1959 Jacini See Col. 1, lines 17–22, 28–36 and 43–52.	4,11
Y	US, A, 4565,696 Published 21 January 1986 Heath et al See col. 3, line 46.	15
Y	US, A, 4,460,577 Published 17 July 1984 Moro et al. See Examples 1, 2 and 5.	16
A	US, A, 4,416,872 Published 22 November 1983 Alving et al.	1–17

* Special categories of cited documents: ¹⁵
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²
26 February 1988	3 0 MAR 1988
International Searching Authority ¹	Signature of Authorized Officer ¹⁰
ISA/US	*Richard D. Lovering* Richard D. Lovering

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	US, A, 4,515,736 Published 07 May 1985 Deamer	1-17
A,P	US, A, 4,684,625 Published 04 August 1987 Eppstein et al.	1-17

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed require-ments to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (May 1986)